Drug manipulation of the PERK-eIF2α pathway post TBI modestly improves visual deficits without preserving retinal ganglion cells

Rohan Bellary[1], Shelby Hetzer[2] (BS, PhD), Dr. Nathan Evanson[3] (MD, PhD)

1. University of Cincinnati, Medical Sciences Undergraduate Program
2. University of Cincinnati, Neuroscience Graduate Program
3. Pediatric Physical Medicine & Rehabilitation, Cincinnati Children's Hospital and Medical Center and Assistant professor, University of Cincinnati

Abstract

Traumatic brain injury (TBI) induces acute primary injuries and long-term injury associated with secondary cellular responses. Traumatic optic neuropathy (TON) is an optic nerve injury that can occur with TBI leading to visual defects, including an impaired optokinetic response (OKR). TBI-induced TON triggers the Endoplasmic Reticulum (ER) stress response with activation of the PERK-eIF2α arm. TON-induced phosphorylation of eIF2α, a downstream ER stress activator, can lead to apoptosis or activate the cell's adaptive unfolded protein response. We hypothesized that dephosphorylation, rather than phosphorylation, of eIF2α would lead to reduced apoptosis and improved visual performance and retinal cell survival. To test this, adult male mice were injected with Salubrinal (increases p-eIF2α, ISRIB (decreases p-eIF2α), or vehicle 60 minutes post-injury then tested for the OKR. Salubrinal improved OKR overall while ISRIB improved only visual acuity in injured mice. Retinal western blot analyses revealed increased p-eIF2α/eIF2α ratio in TBI+Salubrinal alone. RBPMS – a retinal ganglion cell marker – was decreased in all TBI conditions, indicating that neither drug preserved retinal cells. Due to this modest improvement in visual functioning after injury without clear changes in ER stress cascades, further studies are needed to elucidate effects of eIF2α manipulation on adaptive vs. apoptotic cellular responses.

Introduction

Traumatic brain injury (TBI) is caused by blows or trauma to the head or the skull. TBI is a significant health concern that affects approximately 2.9 million people yearly; TBI presents primary injuries that manifest directly and latent secondary effects that tend to cause long-term complications due to pathophysiological changes at the cellular levels, leading to long-term neuronal complications (12). Moreover, the results of TBI are widespread and not limited to any one brain-controlled system (12,13). Optic nerve trauma or traumatic optic neuropathy (TON) is just one pathophysiological outcome associated with TBI. NEVERTHELESS, this TON model poses an opportune system for exploring the effects of TBI and traumatic axonal injury with little known about the mechanisms of TON disease progression or evidence-based treatments available (14). Visual system injury is a significant focus of our lab. TBI leads to visible defects in 50-60% of patients, with 2-3% attributed to TON-specific effects. Due to the concern surrounding this condition and the lack of knowledge around the mechanisms, treatment, or chronic consequences, there is a clear need to discover potential drug interventions.

We previously found that TBI-induced TON leads to elevation of endoplasmic reticulum (ER) stress markers (10,11), specifically through activation of the PERK-eIF2a pathway. It is important to note that there are three branches of ER stress (ATF6, IRE1, and PERK), and for the purpose of this project, we will focus solely on the realm of the PERK pathway. Activation of the PERK-eIF2a pathway in previous studies was associated with functional changes within the visual system as assessed by the optokinetic response test (10,11). In association with changes in optical system function, production of downstream pro-apoptotic factors, such as CHOP (C/EBP Homologous protein), and the cleavage of caspases (Caspase 3 and 12) has been reported during

ER stress. Given that this pathway plays a significant role in TON-induced visual deficits and sustained retinal cellular loss post-TBI, the PERK-eIF2a pathway poses a potential control point in determining the cellular outcomes of retinal cells.

Currently, our hypothesized model follows the idea that a TBI event leads to energy changes within the cell; this causes the ER to dysfunction and leads to a build-up of unfolded proteins (8). The cell will initiate the Unfolded Protein Response (UPR) to restore homeostasis. As shown in **diagram 1**, the UPR leads to the activation of PERK, which can act as a kinase to phosphorylate elongation initiation factor 2a (eIF2a). eIF2a thus poses as a control point within this mechanism as it can shift the cell towards one of two cellular responses – adaptive or apoptotic. Our project examined two drugs, Salubrinal and ISRIB, that have opposing effects on eIF2a but have been dually cited as beneficial within previous blunt models of TBI (7,9). Salubrinal, an inhibitor of eIF2a dephosphorylation, could restore cell functioning by activating the pathway's antioxidant protein translation while simultaneously halting cellular protein translation. ISRIB, an inhibitor of eIF2a phosphorylation, could also reduce ER stress by preventing downstream apoptotic factor translation preventing cell death. Moreover, Salubrinal could also induce cell death by increasing apoptotic factor translation, and ISRIB's prevention of antioxidant defense and protein translation inhibition could equally as likely lead to increased cellular burden and cell death. We aimed to assess the restoration of function in retinal ganglion cells following the administration of two drugs with opposing mechanisms and identify downstream apoptosis markers following injury.

We hypothesized that manipulating the PERK pathway via ISRIB-mediated dephosphorylation of eIF2a would lead to increased RGC survival and improved visual function in TBI-induced TON adult male mice models. Following this, we conversely predicted the opposite effects of Salubrinal intervention.

Materials and Methods

TBI + Drug

To assess visual and retinal function, adult male mice (C57BI/6J) underwent a closed head weight drop model and behavioral assay cited in the previous lab studies (11). TBI occurred on day 0 of experimental procedures. SHAM mice underwent the same procedure before TBI but did not undergo the TBI event itself. Mice were then injected one time with one of three following drugs one-hour post-injury at the following doses – Salubrinal (1.5 mg/kg), ISRIB (2.5 mg/kg), and Vehicle (6.25% DMSO in sterile saline). TBI mice were then evenly split into six cohorts – Salubrinal, ISRIB, or Vehicle intervention plus or minus TBI.

Behavioral Assay

As shown in the vignette in **diagram 3.**,mice underwent five days of behavioral testing to test optomotor function by administering the optokinetic behavioral test using an optomotor machine.The behavioral testing procedure followed a previously described methodology. (11). Optokinetic testing looked to assess the visual response of mice models. A visual response in this task is the involuntary visual tracking of a moving stimulus. Visual acuity is the ability of

a subject to detect distinct visual stimuli (white and black bars in our study)- this was assessed by the cycles/degrees shown on the x-axis of **Fig 1.** and **Fig 2.** Higher contrast (cycles/degrees) was indicative of a less apparent visual discrepancy between the white and black bars utilized within our study

Western Blot Analysis

Mice were euthanized on day seven, and individual retinas were extracted. Retinas were used to produce a protein homogenate that was subsequently utilized for western blot analysis, using a previously described procedure (11). A standard BCA assay was used to determine protein concentrations and subsequent loading amounts. Controls of a non-TBI Brain and water were used within each cohort. Western blot analysis was used to test for semi-quantitative protein expression of downstream PERK-eIF2a markers and included ER stress and apoptotic markers: PERK, eIF2a, P-PERK, P-eIF2a, CHOP, RBPMS (a marker of retinal ganglion cells), Caspase 3, and Caspase 12.

Statistical Analysis

Optokinetic behavioral data were analyzed utilizing a 2-way RM ANOVA within each grating. Western blot data were first analyzed using ImageJ software (15), and factors of interest were compared respectively by normalizing and comparing to total protein. Western blot data were then analyzed using a non-RM 2-way ANOVA. A critical significance level, a, was set at 0.05 for all statistical analyses, with a b = 0.08.

Results

We first began the analysis by observing the effects of drug intervention on visual acuity as well as visual response, as indicated by the optokinetic behavioral assay. Beginning with the ISRIB cohort (**Fig 1.**), we can see that while TBI reduced visual responses as well as visual acuity across all conditions, the TBI + ISRIB condition was significantly improved compared to its control cohort at higher contrast degrees. These data indicate that while ISRIB did not restore all states to baseline, ISRIB was beneficial in helping to modestly restore optomotor function within TBI/TON mice at high levels of the visual grating. The Salubrinal injected cohort (**Fig 2.**) showed a main effect of TBI across both visual acuity and visual. However, in the Salubrinal cohort, visual acuity did not improve at higher contrasts but instead showed a significant improvement in visual responses within optimal rodent levels (16) (0.26 to 0.32 cycles/degree) of contrast. This indicated Salubrinal is beneficial in restoring the overall involuntary visual response. Given the data, both drugs were modestly beneficial in restoring visual functioning in TBI mice.

Western blotting was used to assess the expression of downstream markers. As shown in **Fig 3.** we examined the ratio of P-eIF2a to eIF2a, indicating downstream pathway activation. Data showed that TBI trended towards elevating the ratio in the TBI+Vehicle condition ($p = 0.08$). In the Salubrinal condition, there was a significant increase in the ratio ($p < 0.05$) compared to sham+vehicle. This indicated that Salubrinal was a phosphorylase inhibitor of eIF2a and helped promote downstream phosphorylation/activation of the PERK-eIF2a pathway. ISRIB had no significant effect in its ratio ($p > 0.05$) in TBI vs. control conditions, consistent with being a phosphorylation inhibitor within this system.

RBPMS, a measure of retinal cells, was tested to assess the cellular recovery potential of both therapeutics. As shown in **Fig 4.** TBI had a main effect across all cohorts. TBI significantly reduced levels of RBPMS expression in SHAM, Salubrinal, and ISRIB groups.

CHOP, a downstream ER stress-specific pro-apoptotic marker, was measured to assess the outcomes of downstream PERK pathway activation between the experimental groups. Western blot analysis is shown in **Fig 5.** indicated no significant differences in CHOP expression regardless of TBI condition and drug administration. Salubrinal did not significantly increase the levels of CHOP expression as previously hypothesized within our model. Similarly, ISRIB treatment did not lead to a significant reduction in CHOP. Although the TBI+Vehicle group showed elevated CHOP, this was also not significant.

Western blot analysis of PERK, Cas 3, and Cas 12 expression, as shown in **Fig 5., 6., and 7.** of the supplemental figure section, revealed no significant differences between any cohorts and conditions. TBI had no effect on the downstream expression of any of the listed markers.

Discussion

The optokinetic motor test indicated that both drugs played a role in modestly improving visual functioning to some extent. Data showed that while ISRIB improved visual acuity, Salubrinal improved the overall visual response. This trend in response needs to be noted as well, only looking at RGC's and effects on retinal function; any visual improvement of any kind should be attributed to the Drug's impact on cell type. ISRIB may affect cones rather than rods, which could explain the results; however, more studies need to be completed to further

understand this idea. While neither Drug was able to improve both aspects of vision within the test, both were able to provide some benefit in function, proving that further analysis and studies of these compounds for optic nerve trauma should be continued. Our original hypothesis was correct because ISRIB did improve visual functioning to some extent but was wrong because Salubrinal also provided a beneficial effect.

Western blot analysis painted an interesting picture of our study. Through examining the RBPMS data, we saw that neither Drug helped limit damaged retinal cell counts to pre-TBI conditions. Thus, our hypothesis was wrong because ISRIB did not actually help preserve retinal cell counts. Both drugs were able to restore visual function without restoring the count of retinal cells is significant and points to the possibility of other factors such as complimentary branches of ER stress.

Analysis of the ratio of phosphorylated eIF2a to its standard counterpart, paired with the analysis of CHOP gene expression, posed some of the most intriguing points within this study. The ratio analysis revealed that the ratio was elevated in the TBI + Vehicle condition; however, it was insignificant. When looking at the TBI + Salubrinal condition, we saw a significantly increased ratio of the results. TBI+ISRIB showed a ratio comparable to that of the SHAM+ISRIB condition. Data indicates that Salubrinal did, in fact, help increase the level of p-eIF2a, confirming its function in previously cited studies and its purpose within our hypothesized model. ISRIB followed a similar suit and ~~proved~~ that it was a known phosphorylation inhibitor of eIF2a and supported our original hypothetical model. As the TBI+Vehicle data was not

significant but was elevated to some extent, our original model of apoptotic vs. adaptive pathways seemed to be in line with initial findings in this TON model.

With data indicating that the first half of our model was correct, we expected to see that increased activation of the PERK arm, caused by eIF2a phosphorylation, would lead to a generation of CHOP. CHOP analysis revealed no significant differences across any cohorts, including the TBI+Vehicle and TBI+Salubrinal conditions. Even though Salubrinal proved to have increased eIF2a phosphorylation, the CHOP protein expression level was not elevated. This result confuses our originally proposed hypothesis and poses many important questions. The first one is if our hypothesized PERK pathway activation model is correct; While this model has been used in previous TBI studies, as noted earlier, little has been studied within visual system pathology. It is not unreasonable to question if our model, taken from other non-TON models of TBI, is even applicable to our optical system study. This result's next question lies in the idea that Cas 3 and Cas 12 levels were also not significantly changed. Looking at our RBPMS data, we saw that while neither Drug restored the number of retinal cells, TBI did reduce the number of retinal cells. TBI causes cell death, and caspase expression was expected to be seen post-injury; the lack of significance suggests that ER stress markers were no longer significantly elevated seven days post-injury.

Overall, the data did help us to confirm the function of Salubrinal and ISRIB regarding their effects on eIF2a and their benefit in visual system functioning. Moving forward with this study, the first thing that needs to be reexamined is the dose-response. This study utilized a single dose that occurred one-hour post-TBI. Previous papers have shown that Salubrinal and

ISRIB have half-lives that are less than 48 hours, and the retinas of mice in our study were extracted 7 days after injury/injection occurred. Given this paired with the trend in visual improvement that we observed, an interval dose administration needs to be explored and a higher administered dose.

Next Steps & Limitations

This study was completed originally as a pilot study to help piece together our knowledge of the PERK-eIF2a pathway and hoped to find a potential helpful therapeutic and looked to flesh together with a model of ER stress within the field of TBI induced TON. While the results were limited, data proved significant, and the next steps and limitations, in addition to those already listed, need to be examined.

First, this study was completed only by observing downstream markers of one branch of ER stress- the PERK pathway. As discussed earlier, three known branches of ER stress have been cited in previous TBI-induced models, including the IRE1 and ATF6 branches. Each of these branches contains a set of downstream markers with similar complications to that of PERK and has been shown in previous TBI models to play significant roles in cellular fate and protein translation. Due to time constraints, these branches were not considered within my project but should be examined further in future studies in conjunction with the data discussed within this report.

Next, it should be noted that while cell death markers such as Caspase 3 and Caspase 12 were examined using western blotting during this report, these caspases should be analyzed using

a more accurate enzyme assay. Caspases are proteolytic enzymes, and while western blot analysis is appropriate, an enzyme assay would help gain an idea of Caspase activation rather than simply the quantity of protein present. Similarly, RBPMS was utilized to measure retinal cells within this study; We were interested in RGC function as they are the cells that undergo axonal damage following TBI. An electroretinogram should be implemented to gain a better idea of retinal cell damage to achieve a better idea of effects.

Finally, it should be noted that this study occurred only in adult male mice models. Further studies should examine the effects of TBI-induced TON within a more diverse demographic of animal models, including females and adolescents.

Conclusion

Overall, data suggested that both drugs are modestly beneficial in improving visual functioning in TBI-induced TON in adult male mice without providing retinal cell-saving functions. While we could not gain a complete idea of the mechanisms and the extent of our therapeutic intervention, these drugs should be tested further with consideration of the above limitations and the next steps suggestions.

Figures

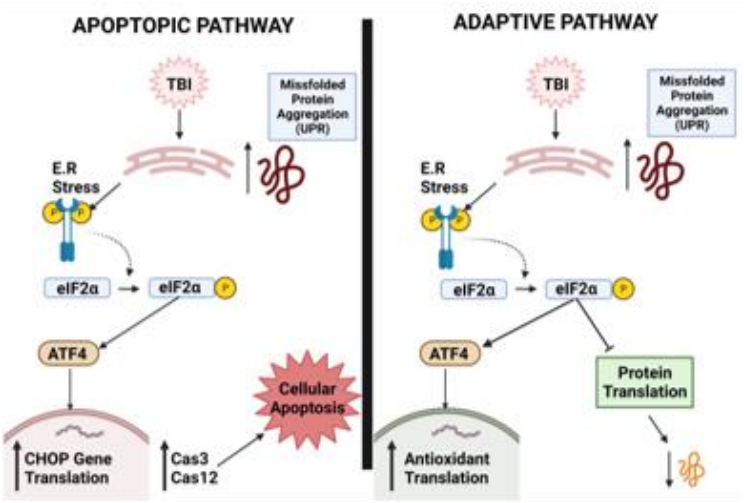
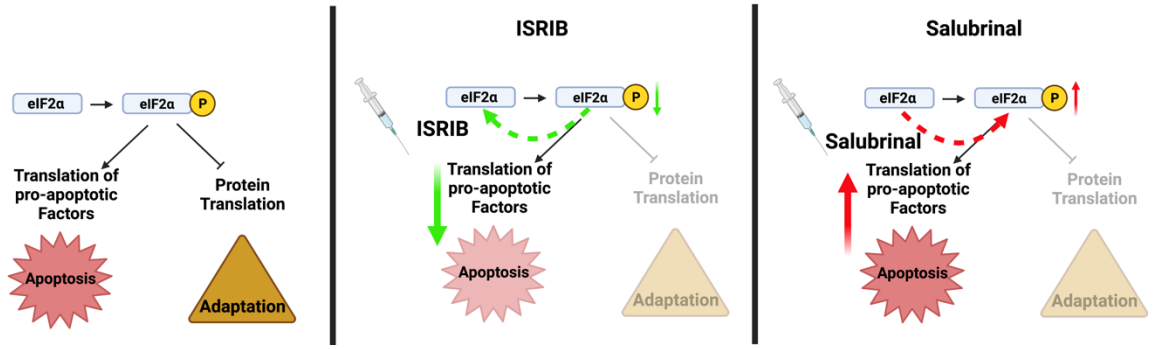


Diagram 1. (Left) Representation of activation of the PERK-eIF2α arm of the ER stress response following a TBI event. Apoptotic markers shown when eIF2α maintains high levels of phosphorylation and diagrams downstream factor production. Adaptive side shows reduction of eIF2α phosphorylation and subsequent reduction of products. Created in Biorender.

Diagram 2. (Bottom) Simplified visual vignette of drug intervention of the PERK pathway. Drug effects shown on eIF2α and hypothesized cellular fates highlighted in figure because of proposed intervention. Created in Biorender.



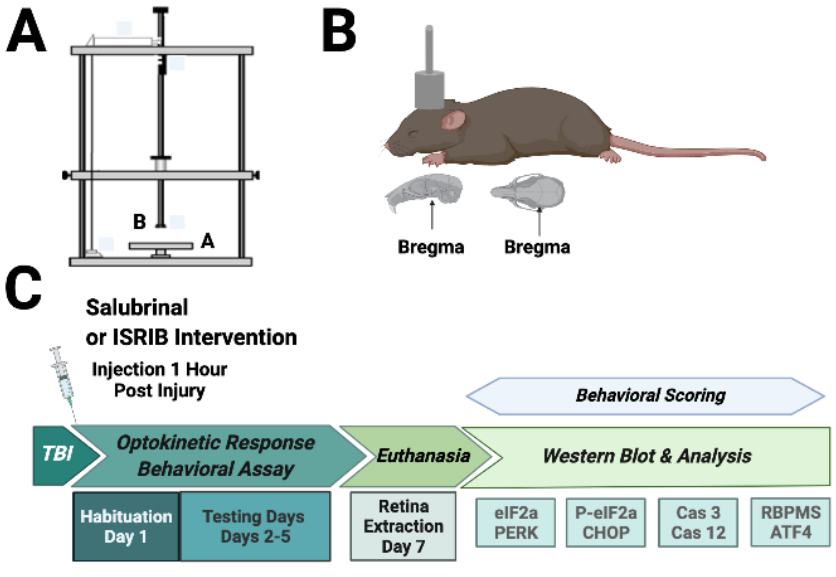


Diagram 3. Visual representation of materials and methods used. Part **A** highlights TBI device used for described TBI procedure. Head was placed on cork plate(A) and the lever (B) was raised to 1.25 cm above bregma. Part **B** displays impact of weight on targeted areas of mice skulls including the bregma region. Mice were anesthetized during procedure and followed standard procedure protocols. Part **C** displays timeline of intervention as well as subsequent testing on animals. Intrapleural injection was used for all drug cohorts; Optokinetic testing utilized a standard optokinetic testing machine as described in previous studies. Western blot analysis aimed to quantify protein expression of boxed factors shown in the diagram.

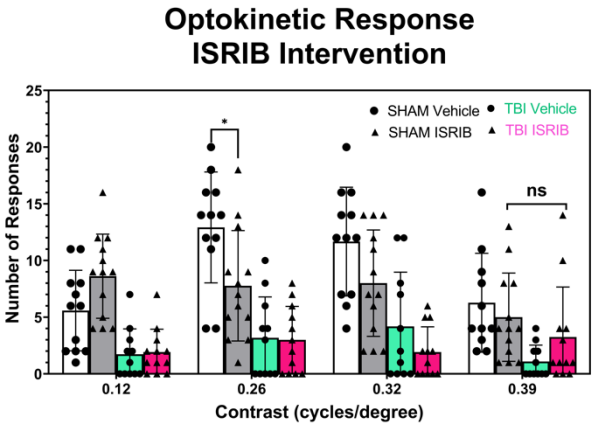
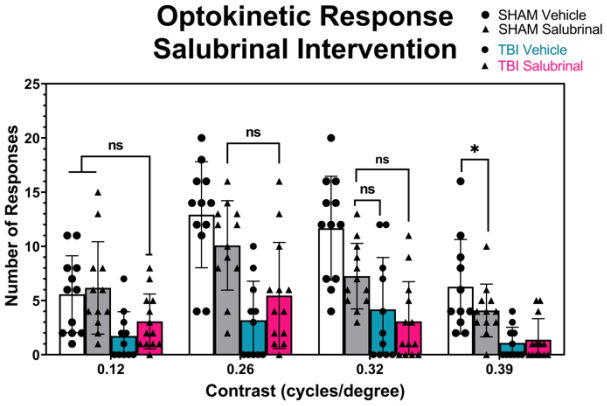
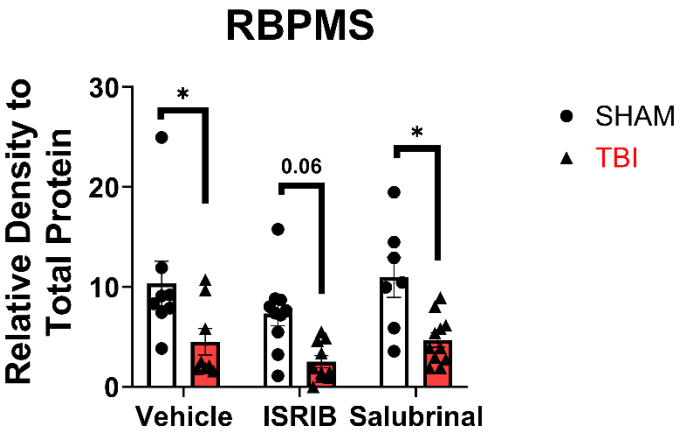


Figure 1. Optokinetic response data of mice followed by ISRIB intervention. A N = 12 was used for all cohorts and a 3-way ANOVA with a=0.05 was used for analysis, all data is mean +/- SEM. Data shows that at higher levels of contrast (cycles/degrees) TBI+ISRIB condition showed a significant improvement in number of visual responses when compared to the TBI + Vehicle condition. Main effect of TBI should be noted in a reduction of responses across all conditions. SHAM + ISRIB saw increased response rate at lower contrasts compared to the SHAM + Vehicle condition. ISRIB modestly improved visual acuity at higher levels of contrast in TBI conditions.

Figure 2. Optokinetic response data of mice followed by Salubrinal intervention. A N= 12 was used for all cohorts and a 3-way ANOVA with a=0.05 was used for analysis, all data is mean +/- SEM. Data shows that at modest/low levels of contrast, Salubrinal + TBI cohorts had higher levels of visual response compared to its control counterpart. Salubrinal intervention showed no effect at higher levels of contrast. Results show modest improvement at low levels of contrast with no consistency as contrast increased; Data thus shows that Salubrinal provided a modest effect in improving overall visual response in certain TBI models.





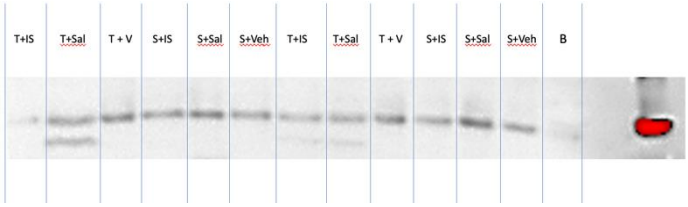


Figure 3. Western blot analysis of RBPMS (marker of healthy RGC's). Western blot shown below graph with conditions labeled. N = 8 for all conditions. 2-way non-RM ANOVA used for all analysis. * = $p < 0.05$. Data is Mean +/- SEM for all sets.. Data shows main effect due to TBI in all cohorts, as expected. No significant recovery was found in the Salubrinal or ISRIB cohort when compared to SHAM + Vehicle control. Results indicate lack of RGC saving function at experimental dose.

Figure 4. Western blot analysis of ratio of P-eIF2a to eIF2a. Western blot shown next to graph of eIF2a and P-eIF2a expression with conditions listed. N=6 for all groups. 2-way non-RM ANOVA used for all analysis. * = $p < 0.05$. Data is Mean +/- SEM for all sets.TBI + Vehicle showed increased ratio compared to SHAM + Vehicle, but not to a level of significance (p=0.08). ISRIB displayed no significant differences between TBI and SHAM cohorts. Salubrinal displayed elevated ratio in the TBI condition compared to SHAM counterpart.

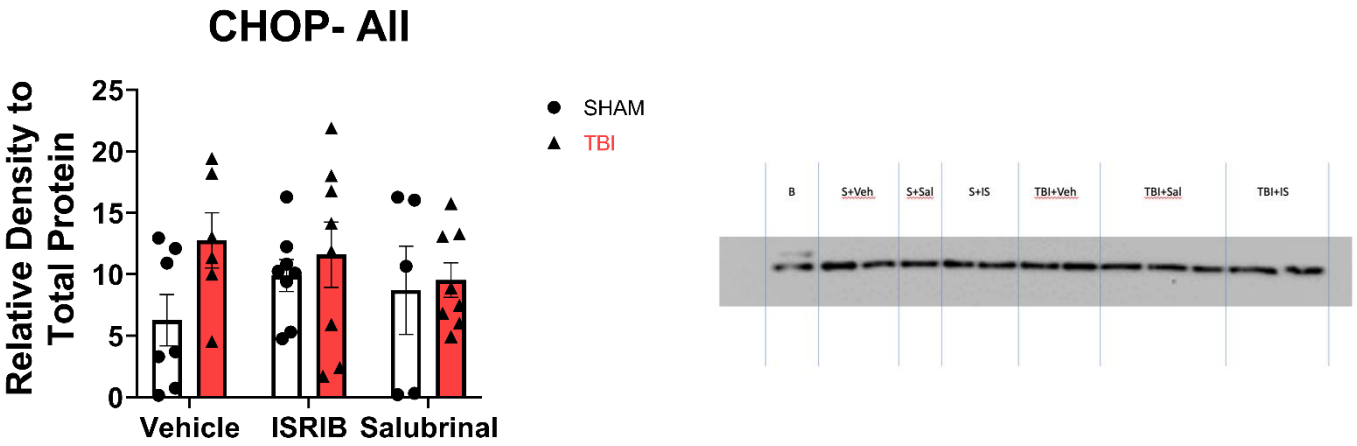


Figure 5. Western blot analysis of CHOP (downstream apoptotic marker). Western blot shown below graph with conditions listed. N= 7 for all conditions. 2-way non-RM ANOVA used for all analysis. * = $p < 0.05$. Data is Mean +/- SEM for all sets.No significant main effect of injury was observed between TBI and SHAM conditions. No significant effect of drug was observed on CHOP elevation or demotion compared to SHAM conditions across both Salubrinal and ISRIB cohorts.

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Supplemental Figures

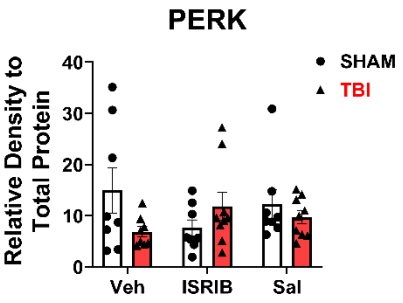

Fig 6. PERK WB Analysis

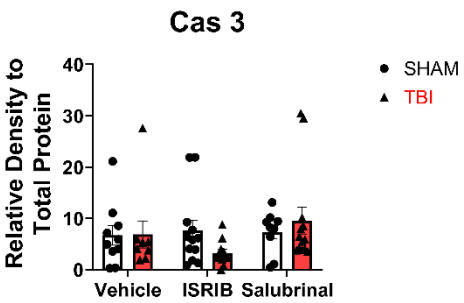

Fig 7. Caspase 3 WB Analysis

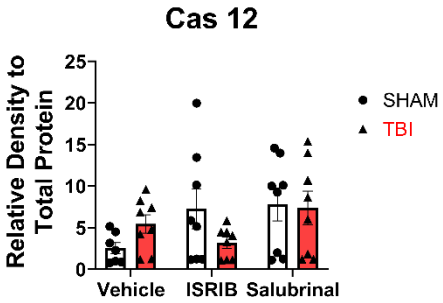

Fig 8. Caspase 12 WB Analysis

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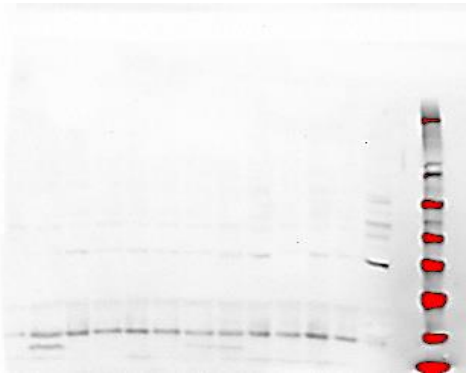

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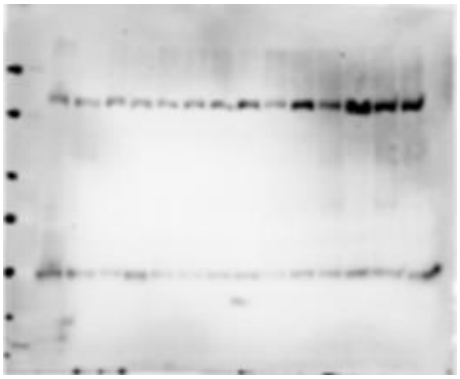

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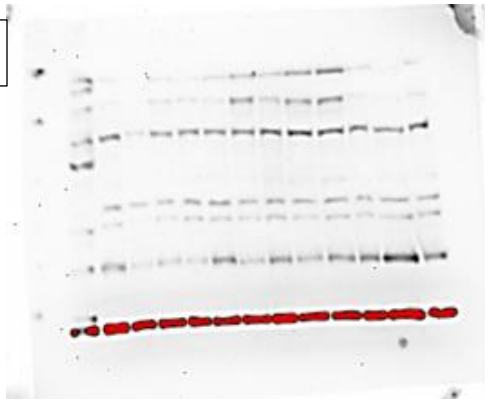

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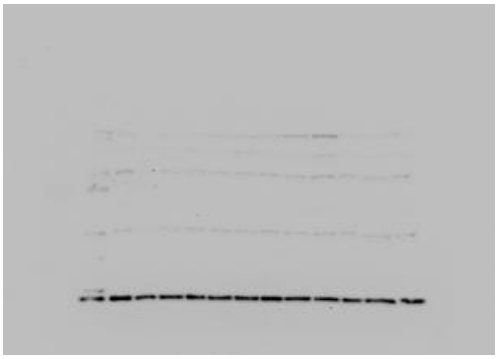

Supplemental Figures: Full Western blots of analyzed protein markers. Key as follows

i. CHOP
ii. RBPMS
iii. eIF2α
iv. P-eIF2α

Author Contributions

The following authors contributed to this project in the following ways and should be acknowledge as such

i. Shelby Hetzer: Optokinetic Behavioral scoring, OMM analysis, Western Blot assistance

ii. Jordyn Torrens: Western Blot procedure and analysis assistance

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